Final
Bacterial Source Tracking on an Unnamed Tributary to Winters Run
Harford County, Maryland

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	analysis of variance
ARA	antibiotic resistance analysis
BST	bacterial source tracking
cfu/100 mL	colony forming units per 100 milliliter
COMAR	Code of Maryland Regulations
EA	EA Engineering, Science, and Technology, Inc.
E. coli	*Escherichia coli*
ENT	*Enterococcus*
EST	estimated count
DUP	duplicate or field duplicate
MDE	Maryland Department of the Environment
MS4	Municipal Separate Storm Sewer System
NPDES	National Pollutant Discharge Elimination System
RCCs	percent correct classification
QA/QC	quality assurance and quality control
µg/ml	microgram per milliliter
U.S. EPA	U.S. Environmental Protection Agency

1. BACKGROUND AND PURPOSE

Since 1998, Harford County has been monitoring baseline water quality conditions on an unnamed tributary to Winters Run. Monitoring at one upstream and one downstream station is being completed as part of the County's ambient water quality monitoring program under the County's NPDES MS4 discharge permit. The upstream station is located at an outfall of an existing stormwater management facility in the Brentwood Park development. The downstream station is located at the Tollgate Road culvert crossing near the Woodland Hills development (**Figure 1-1**).

The drainage area to the downstream station is slightly over twice the size of the drainage area of the upstream station. The two drainage areas consist primarily of medium density residential development, but are quite different in their percentages of land cover, as shown in the Table 1-1 below.

Table 1-1. Percentages of Land Cover

Land Cover	Upstream Station	Downstream Station
Low Density Residential	0 Acres / 0%	35.11 Acres / 21%
Medium Density Residential	49.56 Acres / 63%	56.44 Acres / 33%
High Density Residential	15.27 Acres / 19%	36.96 Acres / 22%
Open Urban	14.19 Acres / 18%	40.58 Acres / 24%
Total Drainage Area	**79.02 Acres**	**169.09 Acres**

Water quality data collected by the County since 1998 indicate that fecal coliform bacteria levels increase during storm events, and that fecal coliform concentrations vary seasonally with concentrations in the summer months that are approximately 10 times higher than during the winter months. Through these monitoring efforts, the County has identified several possible sources of fecal contamination including: sanitary sewer overflows, local sewer lines, septic systems, and domestic animals and wildlife. The purpose of this investigation is to develop and implement a Bacterial Source Tracking (BST) program to help determine the cause(s) of this fecal contamination.

Legend

● Sampling Station

— Stream

002

001

Forest Hill

Bel Air North

24

924

Abingdon

Bel Air South

40

Meters

0   37.5   75   150   225   300

EA Engineering, Science, and Technology, Inc.

Harford County
Bacterial Source Tracking

Harford County, Maryland

N W E S

**Figure 1-1.
Location of
Sampling Stations**

2 November 2009
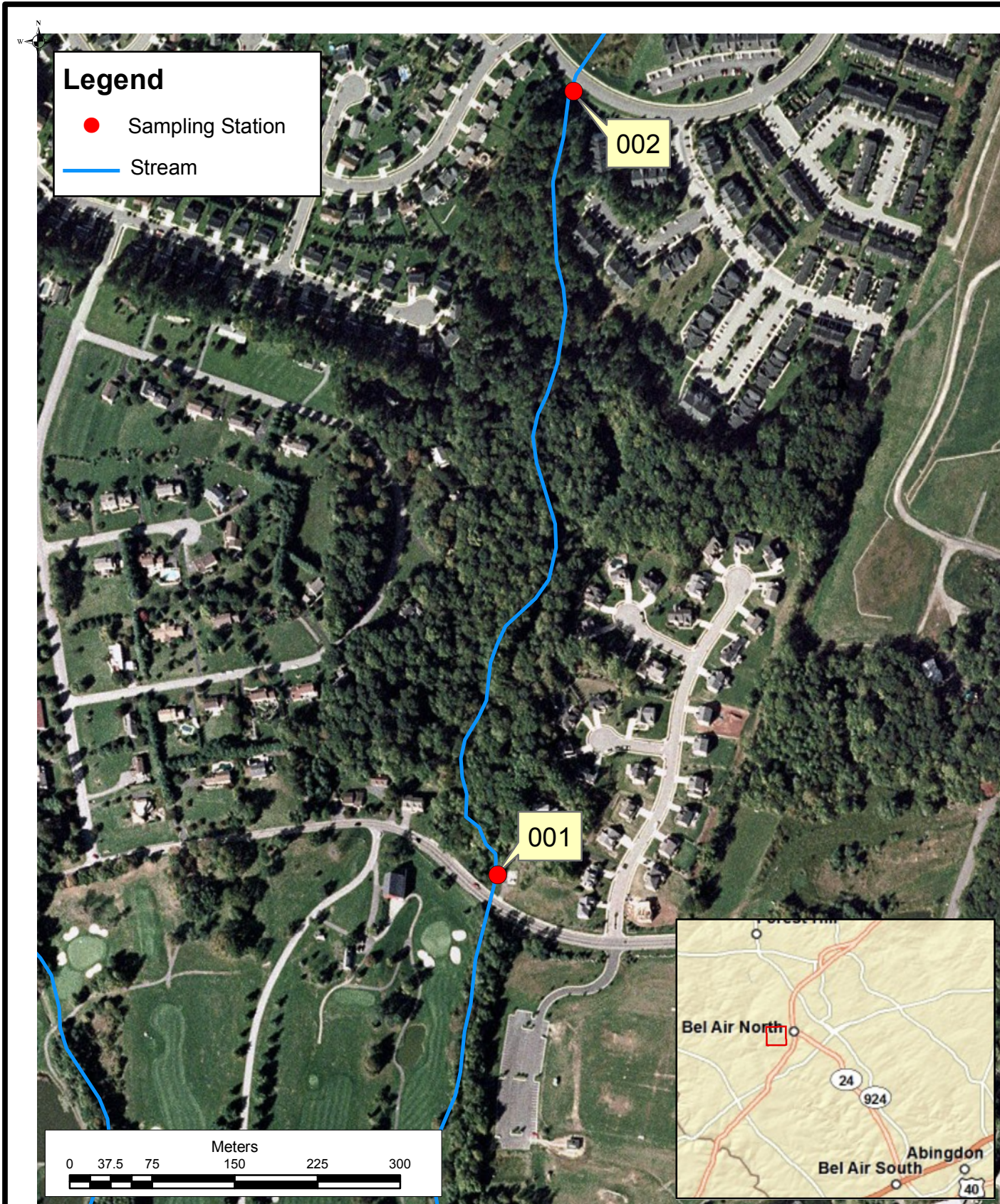
1.1 FECAL-INDICATOR BACTERIA AND BACTERIAL SOURCE TRACKING BACKROUND

1.1.1 Fecal-Indicator Bacteria Testing

Fecal coliforms have been used for many years as indicators for determining the presence of human fecal contamination in surface waters. In recent years, however, alternative indicators including enterococci, *Escherichia coli* (*E. coli*), and *Clostridium perfringens* have been found to better correlate with human pathogens than fecal coliforms. As a result, the U.S. Environmental Protection Agency (U.S. EPA) recommends that enterococci or *E. coli* be used as an indicator for human fecal source instead of fecal coliforms. Similarly, the Maryland Department of the Environment (MDE) has dropped the fecal coliform criteria, and established numerical criteria for enterococci and *E. coli* in Maryland waters (COMAR 26.08.02.03-3).

1.1.2 Bacterial Source Tracking (BST)

Over the past decade, there have been substantial advances in identifying the sources of bacterial pollution to surface waters. BST refers to a group of emerging technologies designed to help determine the sources (the host) of fecal bacteria in environmental samples (i.e., is the source human, pets, livestock, or wildlife).

Numerous BST methods have been developed and published by researchers (U.S. EPA 2005). For this program, Harford County used the Antibiotic Resistance Analysis (ARA) method. The ARA method exposes fecal bacteria to concentrations of various antibiotics and determines the sources of the bacteria (e.g., human, domestic animal, wildlife, and livestock) based on how strongly the bacteria resist specific antibiotics. The assumption is that fecal bacteria from human hosts will have a significantly greater resistance to certain antibiotics (e.g. penicillin, erythromycin) than will bacteria from domestic animals or wildlife hosts, based upon the selective pressure of microbial populations found in the gastrointestinal tract of those hosts. Fecal bacteria from collected aqueous samples are cultured in a series of agar plates, with each plate having a specific antibiotic concentration. Following a specific incubation period, fecal

colonies are reported and scored by the increase or decrease of bacteria. By comparing the results to a regional specific "library" of fecal results from resident host species, the ARA method is able to determine the relative contribution of bacterial sources in collected samples.

2. METHODS

2.1 SAMPLING LOCATIONS

Sampling was conducted at two stations on the unnamed tributary to Winters Run (**Figure 1-1**). The location of these stations was chosen to coincide with the County's ambient water quality monitoring program.

2.2 SAMPLING METHODOLOGY

The BST study was designed to investigate dry weather and wet weather conditions, as well as seasonality. Beginning in November 2007, surface water samples were collected during dry and wet weather conditions through December 2008. A known-source library was developed consisting of sewage samples and animal scat collected from within or nearby the watershed.

2.2.1 Dry Weather Surface Water Samples

Dry weather samples were collected approximately monthly by Harford County personnel. For this study, dry weather was defined as no measurable rainfall within a within the preceding 72-hours.

2.2.2 Wet Weather Surface Water Samples

Wet weather sampling was conducted by EA Engineering, Science, and Technology, Inc. (EA). A wet weather event was defined as at least 0.1 inch of rainfall within a 24-hour period. The wet weather samples consisted of a single grab sample collected within 24-hours following the end of the storm.

2.2.3 Procedure for Collection of Surface Water Samples

Sterilized 150-mL Nalgene® sample bottles were supplied by the Salisbury University BST laboratory. Each bottle was labeled with a Sample ID that identifies the sample location, and sample type such as normal (N) or field duplicate (DUP). Water was

collected below the water surface with the open end of the sterilized sample bottle directed upstream.

Samples were immediately stored on ice, and then shipped by FedEx® for morning delivery to the BST laboratory. In order to insure that sample holding times did not exceed 24-hours, all surface water samples were collected and shipped during the normal business hours of the FedEx® shipping office.

A Chain of Custody/Data Sheet was submitted to the laboratory along with the samples.

2.2.4 Scat and Sewage Sampling

Scat samples for the development of a regional known source library were collected within the watershed by EA. Scat samples consisted of approximately 5 to 10 grams of scat, and were placed into sterilized zip lock bags. The location of the scat sample was recorded with GPS, and the animal source of the scat samples was identified by EA biologists (e.g., human, dog, horse, deer, fox, rabbit, and goose). Scat samples were stored on ice for delivery to the BST laboratory.

Human sewage samples, also required for the regional known-source library, were collected from the Sod Run Wastewater Treatment Plant by Harford County personnel. Sewage samples were stored on ice, and shipped by FedEx for overnight delivery to the BST laboratory.

A Chain of Custody/Data Sheet was submitted to the laboratory along with the scat and sewage samples.

2.3 LABORATORY METHODS

Laboratory analysis was conducted at the Salisbury University BST laboratory under the direction of Dr. Mark Frana and Dr. Elichia Venso. Dr. Frana's laboratory has been conducting BST investigations for MDE for several years, and consistency is important for the acceptance of the BST methodology and interpretation of results.

2.3.1 Isolation of *Enterococcus* from Known-Source Samples

Fecal material from the scat samples was suspended in phosphate buffered saline, and then plated onto selective *m-Enterococcus* agar. After incubation at 37° C, up to eight (8) *Enterococcus* isolates were randomly selected from each plate for ARA testing.

2.3.2 Isolation of *Enterococcus* from Surface Water Samples

Bacterial isolates were collected by membrane filtration. Up to 24 randomly selected *Enterococcus* isolates were collected from each surface water sample and all isolates were evaluated by the BST laboratory.

2.3.3 Antibiotic Resistance Analysis

Each bacterial isolate from both water and scat were grown in Enterococcosel$^{®}$ broth (Becton Dickinson, Sparks, MD) prior to ARA testing. *Enterococci* are capable of hydrolyzing esculin, turning this broth black. Only esculin-positive isolates were tested for antibiotic resistance.

Bacterial isolates were plated onto tryptic soy agar plates, each containing a different concentration of a given antibiotic. Plates were incubated overnight at 37°C and isolates then scored for growth (resistance) or no growth (sensitivity). Data consisting of a "1" for resistance or "0" for sensitivity for each isolate at each concentration of each antibiotic were then entered into a spread-sheet for statistical analysis. **Table 2-1** includes the antibiotics and concentrations currently used as isolates in analyses for this watershed.

Table 2-1. Antibiotics and concentrations used for ARA

Antibiotic	Concentration (µg/mL)
Amoxicillin	0.625
Cephalothin	10, 15, 30, 50
Chloramphenicol	10
Chlortetracycline	60, 80, 100
Erythromycin	10
Gentamycin	5, 10, 15
Neomycin	40, 60, 80
Oxytetracycline	20, 40, 60, 80, 100
Salinomycin	10
Streptomycin	40, 60, 80, 100
Tetracycline	10, 30, 50, 100
Vancomycin	2.5

2.3.4 KNOWN-SOURCE LIBRARY

Enterococcus isolates were obtained from human sewage and animal scat samples
from known sources (e.g., human, dog, horse, deer, fox, rabbit, and goose) in the
watershed. A library of patterns of *Enterococcus* isolate responses to the panel of
antibiotics was developed and analyzed using the statistical software CART® (Salford
Systems, San Diego, CA). *Enterococcus* isolate response patterns were also obtained
from bacteria in water samples collected at the monitoring stations. Statistical pattern
matching techniques were used to compare the *Enterococcus* isolate response patterns
to those in the regional known source library in order to identify the probable source of
each water isolate.

For this study, 637 known-source isolates were collected and used for the regional
know-source library.

2.4 STATISTICAL ANALYSIS

Scientists from the BST laboratory applied a tree classification method CART®, to build a model that classified isolates into source categories based on ARA data (Hastie et al 2001). CART® builds a classification tree by recursively splitting the library of isolates into two nodes. Each split is determined by the antibiotic variables (antibiotic resistance measured for a collection of antibiotics at varying concentrations). The first step in the tree-building process splits the library into two nodes by considering every binary split associated with every variable. The split is chosen that maximizes a specified index of homogeneity for isolate sources within each of the nodes. In subsequent steps, the same process is applied to each resulting node until a *stopping* criterion is satisfied in which all isolates in the node are from the same source. Nodes where an additional split would lead to only an insignificant increase in the *homogeneity index* relative to the *stopping* criterion are referred to as *terminal* nodes. A split that achieves the theoretical maximum for homogeneity would produce two nodes each containing library isolates from only one source.

The collection of *terminal* nodes defines the classification model. Each *terminal* node is associated with one source, the source isolate with an unknown source, based on the most populous source among the library isolates in the node.

For each water sample isolate, its antibiotic resistance pattern was identified with one specific *terminal* node and was assigned the source of the majority of library isolates in that *terminal* node. The CART® tree-classification method employed includes various features to ensure the development of an optimal classification model.

2.5 QUALITY CONTROL SAMPLES

2.5.1 Field Duplicates

Approximately 15 percent of the field-collected water samples were field duplicates. Each field duplicate was collected at the same time and location as the initial surface water field sample.

2.5.2 Laboratory Replicates

Variability between test replicates was monitored by retesting 10 percent of the number of field samples per batch as recommended by U.S. EPA (2005).

2.5.3 Field Blanks

In accordance with U.S. EPA guidance (U.S. EPA, 2005), approximately 5 percent of all field-collected samples were field blanks. Field blanks consist of sterile water placed into a laboratory sample bottle. Field blanks were submitted to the laboratory along with the regular samples.

2.5.4 Method Controls

To verify that no contamination is introduced during the process of sample collection and analysis, both positive and negative controls were included with each sample batch.

3. RESULTS AND DISCUSSION

3.1 Known-Source Library

Fecal samples (scat) and human sewage samples were collected over the course of the study from October 2007 through December 2008. As summarized in **Table A-1 (Appendix A)**, there were 68 scat samples collected and identified as deer, goose, fox, raccoon, horse, dog, rabbit, or passerine bird. In addition, there were 17 human sewage samples collected. In total, the scat and sewage samples yielded 637 known-source isolates that were grouped into four categories: human, livestock (horse), pet (dog), and wildlife (deer, fox, goose, rabbit, raccoon). The library was tested for its ability to correctly classify a portion of randomly chosen isolates from the library when treated as unknowns. Average rates of correct classification for the library were determined by repeating this process with different probability thresholds. A sample was considered to be correctly classified if a minimum threshold of correct classification was achieved for the isolates grown from that sample. For example, a threshold probability of 60% means that a sample is considered to be correctly classified if at least 60% of the isolates grown from that sample are correctly classified. A high threshold probability is necessary to insure a high rate of correct classification, however, a high threshold also results in a higher rate of unknowns, which are samples that cannot be classified.

For this study, a CART® threshold probability of 70% was shown to yield the optimal combination of correct classification and unknowns, with an overall rate of correct classification of 87.5% with 23.4% unknowns. The know-source library was able to correctly classify 88% of the isolates from human sewage samples, followed by 83% for pet scat, 69% for wildlife scat, and 50% for livestock scat (**Table 3-WIN Appendix B**). A detailed discussion of statistical methodology and results of the analysis of isolates from the known-source library is presented in Frana and Venso (2009) (**Appendix B**).

The high rate of correct classification for human *Enterococcus* spp. demonstrates that the regional known-source library can be used to reliably detect the presence of human

fecal contamination in the surface waters of the Winters Run watershed. The classification of specific non-human sources was found to be more problematic. Therefore, the regional known-source library is best used as a tool for differentiating between human and non-human sources of fecal bacteria.

3.2   Surface Water Samples

Surface water sampling was conducted from November 2007 through December 2008. During that period, there were 17 sampling events consisting of nine dry weather events and eight wet weather events as summarized in **Table 3-1**.

Table 3-1. Samples collected for BST analysis

Sample Date	Wet or Dry Weather	24-Hour Rainfall (inches)	72-Hour Rainfall (inches)
11/15/2007	Wet	0.88	1.46
11/29/2007	Dry	0	0
12/19/2007	Dry	0	0
1/31/2008	Dry	0	0.12
2/28/2008	Dry	0	0.16
3/5/2008	Wet	0.49	0.52
3/27/2008	Wet	0.03	0.03
4/29/2008	Wet	0.86	1.31
5/13/2008	Wet	0.06	1.23
5/22/2008	Wet	0.02	0.57
6/26/2008	Dry	0	0
7/23/2008	Wet	0.03	0.2
8/28/2008	Dry	0	0
9/23/2008	Dry	0	0
11/20/2008	Dry	0	0.03
12/10/2008	Wet	0.24	0.24
12/18/2008	Dry	0	0.74

3.2.1 Probable Host Source

A total of 931 *Enterococcus* isolates from the surface water samples were analyzed by CART® statistical analysis. The CART® statistical analysis was able to classify 656

isolates, which equates to 70% of the isolates from the surface water samples. The average probable host source of the isolates is presented in **Table 3-2**, along with the relative percent contribution that does not include unclassified isolates. Wildlife was found to comprise 43% percent of the classified isolates, followed by 30% human, 18% pet, and 9% livestock. The 9% livestock as a potential source is interesting, because there is no livestock residing within the watershed. A plausible explanation of the livestock isolates is from the use of cow or horse manure as a garden fertilizer.

Table 3-2. Percent isolates classified and relative contribution of classified isolates by probable source

Source	Percent Classification	Relative Percent Contribution of <u>Classified Isolates</u> [1]
Wildlife	30	43
Human	21	30
Pet	13	18
Livestock	6	9
Unclassified[1]	30	

1. The Relative Percent Contribution does not include unclassified isolates.

3.2.2 Comparison of Replicate Samples

Table 3-3 presents a comparison of replicate samples. For *Enterococcus* , little variance was found between replicate samples.   For the predicted source contributions, however, there was significant variability in the replicate samples. This variability is caused from the subsampling of 24 randomly chosen isolates from each sample. This illustrates the importance of basing conclusions of probable sources on multiple samples spaced over time, and not on any individual water sample.

Table 3-3. Comparison of replicate samples

Station	Sample Date	Sample Type [1]	ENT [2] (cfu/100mL)	Percent Predicted Classification				
				Human	Wildlife	Pet	Livestock	Unknown
001	3/5/2008	N	130	29	42	13	8	8
001	3/5/2008	DUP	160	29	25	17	0	29
001	5/13/2008	N	163	8	25	17	17	33
001	5/13/2008	DUP	180	8	25	13	29	25
001	6/26/2008	N	173	21	38	4	0	38
001	6/26/2008	DUP	195	0	63	0	8	29
002	11/15/2007	N	8000	29	42	17	8	4
002	11/15/2007	DUP	8000	21	42	33	0	4
002	11/29/2007	N	10	8	17	54	8	13
002	11/29/2007	DUP	14	23	9	41	0	27
002	6/26/2008	N	210	17	25	4	13	42
002	6/26/2008	DUP	350	21	38	8	4	29

[1] N = parent sample; DUP = duplicate; [2] ENT = *Enterococcus*

3.2.3 *Enterococcus* Density Results

Total concentrations of *Enterococcus* fecal bacteria are presented in **Table 3-4**. A summary of *Enterococcus* bacteria concentrations is provided in **Table 3-5**. The geometric mean concentration of *Enterococcus* bacteria were very similar between station 001 and 002, being 95 and 97 cfu/100 mL, respectively. These concentrations exceed the MDE's steady state geometric mean water quality standard of 33 cfu/100 mL for a Tier 3 beach (MDE, 2003). *Enterococcus* concentrations also frequently exceed the single sample maximum criteria of 78 cfu/100 mL for a Tier 3 beach (MDE, 2003). The frequent exceedance of *Enterococcus* water quality standards is evidence of human fecal contamination in the watershed.

Table 3-4. Concentrations of total *Enterococcus* found in water samples

Sample Date	Station	Sample Type	Result
11/15/2007	001	N	>5000
11/15/2007	002	DUP	>8000
11/15/2007	002	N	>800
11/29/2007	001	N	9 EST
11/29/2007	002	DUP	14
11/29/2007	002	N	10
12/19/2007	001	N	70
12/19/2007	002	N	62
1/31/2008	001	N	9 EST
1/31/2008	002	N	16
2/28/2008	001	N	5 EST
2/28/2008	002	N	9 EST
3/5/2008	001	DUP	160
3/5/2008	001	N	130
3/5/2008	002	N	157
3/27/2008	001	N	9 EST
3/27/2008	002	N	5 EST
5/13/2008	001	DUP	180
5/13/2008	001	N	163
5/13/2008	002	N	240
5/22/2008	001	N	32
5/22/2008	002	N	220
6/26/2008	001	DUP	195
6/26/2008	001	N	173
6/26/2008	002	DUP	350
6/26/2008	002	N	210
7/23/2008	001	N	480
7/23/2008	002	N	230

Table 3-4. Concentrations of total *Enterococcus* found in water samples

Sample Date	Station	Sample Type	Result
8/28/2008	001	N	200
8/28/2008	002	N	40
9/23/2008	001	N	113
9/23/2008	002	N	17
11/20/2008	001	N	117
11/20/2008	002	N	23
12/10/2008	001	N	510
12/10/2008	002	N	900
12/18/2008	001	N	53
12/18/2008	002	N	90

N = parent sample; DUP = duplicate; EST = estimated

Table 3-5. Summary of total *Enterococcus* bacteria concentrations at stations 001 and 002

	Station 001	Station 002
N	17	17
Geomean	95	97
Minimum	5	5
25th Percentile	32	17
Median	117	90
75th Percentile	200	240
Maximum	5,000	8,000

3.2.4 Effects of Antecedent Rainfall on Bacteria Density and Probable Sources

An analysis of variance (ANOVA) was conducted to investigate the effects of station location, seasonality and antecedent rainfall on the concentration and potential sources of *Enterococcus* fecal bacteria. The ANOVA was performed on the log-transformed *Enterococcus* concentrations, and then means comparisons were conducted on significant main effects using Fisher's Least Significant Difference at the 95% significance level.

The results of the ANOVA on *Enterococcus* concentrations showed that station location was not a significant factor. The month of the year and antecedent rainfall, however, were both found to be factors at the 95% significance level. The highest *Enterococcus*

concentrations were measured in June and July, while the lowest concentrations were found in January and February (**Table 3-6**). Both the 24- and 72-hour antecedent rainfall were highly associated with higher *Enterococcus* concentrations ($p < 0.0001$), with the 72-hour rainfall being the better predictor of high *Enterococcus* concentration. The relative source contribution of bacteria (i.e., percent human, percent wildlife, etc.), however, was not significantly affected by rainfall. This indicates that both human and non-human bacteria loads increase during wet weather. Higher non-human bacteria loads are expected during wet weather due to increased surface runoff. However, the observed higher human bacteria load during wet weather is consistent with leaking septic system(s) or a failing sanitary sewer system that can be infiltrated by rainfall causing spillover into the watershed during wet weather events.

Table 3-6. Geomean of total *Enterococcus* bacteria concentrations by month

Month	Number of Samples	Geomean (cfu/100 mL)	Fisher's Least Significant Difference Grouping [1]
July	2	332	A
June	4	223	BA
November	8	180	BA
December	6	146	BA
May	5	138	BA
August	2	89	BA
September	2	44	CB
March	5	43	CB
January	2	12	C
February	2	7	C

1. Means with common letters are not different at the 95% significance level.

4. SUMMARY AND CONCLUSIONS

This report presents the results of a Bacterial Source Identification (BST) study conducted in the surface water of an unnamed tributary to Winters Run in Harford County, Maryland from November 2007 through December 2008. The BST study was conducted on *Enterococcus* isolates using Antibiotic Resistance Analysis (ARA), in which antibiotic resistance patterns in unknown isolates are matched to a regional known-source library consisting of isolates from human sewage and animal scat. The results of the study showed that wildlife was the largest contributor of fecal bacteria comprising on average 43% of the classified isolates. The human source was the second largest contributor at 30% of the classified isolates, followed by 18% pet, and 9% livestock. The average total *Enterococcus* concentration was about 100 cfu/100 mL, which exceeds the Maryland Department of the Environment steady state water quality standard of 33 cfu/100 mL for a tier 3 recreational beach. The 72-hour antecedent rainfall was found to be the best predictor of high total *Enterococcus* concentration, with heavy rainfall increasing *Enterococcus* concentrations by one to two orders of magnitude. The surface water samples collected in June and July had the highest average *Enterococcus* concentrations, while samples collected in January and February had the lowest. This BST study shows relatively high concentration of *Enterococcus*, of which approximately 30% may be attributed to human fecal pollution, such as sanitary sewer leaks or failing septic systems within the watershed.

5. LITERATURE CITED

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APPENDIX A

SCAT AND SEWAGE SAMPLES COLLECTED FOR KNOWN-SOURCE LIBRARY

Table A-1. Scat and sewage samples collected for known-source library

Event Date	Sample ID	Source
10/18/2007	WIN01	deer
10/18/2007	WIN02	deer
10/18/2007	WIN03	deer
10/18/2007	WIN04	raccoon
10/18/2007	WIN05	dog
10/18/2007	WIN06	goose
12/18/2007	WIN07	sewage
4/2/2008	WIN08	deer
4/2/2008	WIN09	deer
4/2/2008	WIN010	deer
4/2/2008	WIN011	bird
4/2/2008	WIN012	fox
4/2/2008	WIN013	deer
4/2/2008	WIN014	deer
4/2/2008	WIN015	goose
4/2/2008	WIN016	goose
4/2/2008	WIN017	goose
4/2/2008	WIN018	goose
4/2/2008	WIN019	goose
4/2/2008	WIN020	goose
4/2/2008	WIN021	goose
4/2/2008	WIN022	dog
4/2/2008	WIN023	dog
4/2/2008	WIN024	dog
4/2/2008	WIN025	horse
4/29/2008	WIN026	sewage
6/12/2008	WIN027	goose
6/12/2008	WIN028	goose
6/12/2008	WIN029	goose
6/12/2008	WIN030	dog
6/12/2008	WIN031	dog
6/12/2008	WIN032	dog
6/12/2008	WIN033	horse
6/12/2008	WIN034	horse
6/12/2008	WIN035	horse
6/12/2008	WIN036	deer
6/12/2008	WIN037	deer
6/12/2008	WIN038	deer
7/27/2008	WIN039	deer

Table A-1. Scat and sewage samples collected for known-source library

Event Date	Sample ID	Source
6/12/2008	WIN040	deer
6/12/2008	WIN041	deer
6/12/2008	WIN042	deer
6/12/2008	WIN043	deer
6/12/2008	WIN044	raccoon
6/12/2008	WIN045	goose
6/12/2008	WIN046	goose
6/12/2008	WIN047	dog
6/12/2008	WIN048	dog
6/12/2008	WIN049	horse
6/12/2008	WIN050	horse
6/12/2008	WIN051	horse
6/12/2008	WIN052	goose
6/12/2008	WIN053	goose
8/14/2008	WIN054	sewage
8/14/2008	WIN055	sewage
8/14/2008	WIN056	sewage
8/26/2008	WIN057	sewage
8/26/2008	WIN058	sewage
8/26/2008	WIN059	sewage
8/17/2008	WIN060	raccoon
8/17/2008	WIN061	raccoon
8/17/2008	WIN062	dog
8/24/2008	WIN063	raccoon
8/24/2008	WIN064	raccoon
8/24/2008	WIN065	raccoon
8/24/2008	WIN066	fox
8/24/2008	WIN067	fox
8/24/2008	WIN068	fox
9/24/2008	WIN069	sewage
9/24/2008	WIN070	sewage
9/24/2008	WIN071	sewage
9/29/2008	WIN072	deer
9/29/2008	WIN073	deer
9/29/2008	WIN074	dog
9/29/2008	WIN075	dog
9/30/2008	WIN076	goose
9/30/2008	WIN077	goose
10/2/2008	WIN078	fox

Table A-1. Scat and sewage samples collected for known-source library

Event Date	Sample ID	Source
10/2/2008	WIN079	fox
10/28/2008	WIN080	sewage
10/28/2008	WIN081	sewage
10/28/2008	WIN082	sewage
12/02/2008	WIN083	sewage
12/02/2008	WIN084	sewage
12/02/2008	WIN085	sewage

APPENDIX B

ANALYTICAL REPORT FROM SALISBURY UNIVERSITY BST LABORATORY

Final Report

EA Engineering, Science, and Technology, Inc.

Identifying Sources of Fecal Pollution in an Unnamed Tributary of Winters Run Watershed, Harford County, Maryland

October 2007 – December 2008

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INTRODUCTION

Microbial Source Tracking. Microbial Source Tracking (MST) is a relatively recent scientific and technological innovation designed to distinguish the origins of enteric microorganisms found in environmental waters. Several different methods and a variety of different indicator organisms (both bacteria and viruses) have successfully been used for MST, as described in reviews (Scott *et al.*, 2002; Simpson *et al.*, 2002; Field and Samadpour, 2007). When the indicator organism is bacteria, the term Bacterial Source Tracking (BST) is often used. Some common bacterial indicators for BST analysis include: *E. coli, Enterococcus* spp., *Bacteroides-Prevotella,* and *Bifidobacterium* spp.

Techniques for MST can be grouped into one of the following three categories: molecular (genotypic) methods, biochemical (phenotypic) methods, or chemical methods. Ribotyping, Pulsed-Field Gel Electrophoresis (PFGE), and Randomly-Amplified Polymorphic DNA (RAPD) are examples of molecular techniques. Biochemical methods include Antibiotic Resistance Analysis (ARA), F-specific coliphage typing, and Carbon Source Utilization (CSU) analysis. Chemical techniques detect chemical compounds associated with human activities, but do not provide any information regarding nonhuman sources. Examples of this type of technology include detection of optical brighteners from laundry detergents or caffeine (Simpson *et al*., 2002, Field and Samadpour, 2007).

Many of the molecular and biochemical methods of MST are "Library-based," requiring the collection of a database of fingerprints or patterns obtained from indicator organisms isolated from known sources. Statistical analysis determines fingerprints/patterns of known sources species or categories of species (*i.e.*, human, livestock, pets, wildlife). Indicator isolates collected from water samples are analyzed using the same MST method to obtain their fingerprints or patterns, which are then statistically compared to those in the Library. Based upon this comparison, the final results are expressed in terms of the "statistical probability" that the water isolates came from a given source (Price *et al.* 2006; Simpson *et al.* 2002).

In this BST project, we studied the watershed of an unnamed tributary of Winters Run (Winters Run Watershed) in Harford County, Maryland. The methodology used was the ARA with *Enterococcus* spp. as the indicator organism. Previous BST publications have demonstrated the predictive value of using this particular technique and indicator organism (Price *et al*., 2006, 2007; Simpson *et al.* 2002; Hagedorn, 1999; Wiggins, 1999).

Antibiotic Resistance Analysis. A variety of different host species can potentially contribute to the fecal contamination found in natural waters. Many years ago, scientists speculated on the possibility of using resistance to antibiotics as a way of determining the sources of this fecal contamination (Bell *et al.*, 1983; Krumperman, 1983). In ARA, the premise is that bacteria isolated from different hosts can be discriminated based upon differences in the selective pressure of microbial populations found in the gastrointestinal tract of those hosts (humans, livestock, pets, wildlife) (Wiggins, 1996). Microorganisms isolated from the fecal material of wildlife would be expected to have a much lower level of resistance to antibiotics than isolates

collected from the fecal material of humans, livestock and pets. In addition, depending upon the specific antibiotics used in the analysis, isolates from humans, livestock and pets could be differentiated from each other.

In ARA, isolates from known sources are tested for resistance or sensitivity against a panel of antibiotics and antibiotic concentrations. This information is then used to construct a Library of antibiotic resistance patterns from known-source bacterial isolates. Microbial isolates collected from water samples are then tested and their resistance results are recorded. Based upon a comparison of resistance patterns of water and Library isolates, a statistical analysis can predict the likely host source of the water isolates (Price *et al*., 2006; Wiggins 1999).

LABORATORY METHODS

Isolation of *Enterococcus* from Known-Source Samples. Fecal samples, identified to source, were shipped overnight to the Salisbury University (SU) BST lab by EA Engineering, Science, & Technology, Inc. (EA Engineering) personnel. Fecal material suspended in phosphate buffered saline was plated onto selective DIFCO™ m-*Enterococcus* agar. After incubation at 37° C, up to eight (8) *Enterococcus* isolates were randomly selected from each fecal sample for ARA testing.

Isolation of *Enterococcus* from Water Samples. Water samples were collected by EA Engineering staff and shipped overnight to the SU BST lab. Bacterial isolates were collected by membrane filtration onto DIFCO™ m-*Enterococcus* agar. Up to 24 randomly selected *Enterococcus* isolates were collected from each water sample.

Antibiotic Resistance Analysis. Each bacterial isolate from both water and scat were grown in Enterococcosel[®] broth (Becton Dickinson, Sparks, MD) prior to ARA testing. *Enterococci* are capable of hydrolyzing esculin, turning this broth black. Only esculin-positive isolates were tested for antibiotic resistance.

Bacterial isolates were plated onto tryptic soy agar plates, each containing a different concentration of a given antibiotic. Plates were incubated overnight at 37° C and isolates then scored for growth (resistance) or no growth (sensitivity). Data consisting of a "1" for resistance or "0" for sensitivity for each isolate at each concentration of each antibiotic was then entered into a spread-sheet for statistical analysis.

The following table includes the antibiotics and concentrations used for isolates in analyses for the study watershed.

Table 1. Antibiotics and concentrations used for ARA.
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Antibiotic	Concentration (µg/ml)
Amoxicillin	0.625
Bacitracin	25, 50
Cephalothin	10, 15, 30, 50
Chloramphenicol	10
Chlortetracycline	60, 80, 100
Erythromycin	10
Gentamycin	5, 10, 15
Kanamycin	25, 50
Neomycin	40, 60, 80
Oxytetracycline	20, 40, 60, 80, 100
Streptomycin	40, 60, 80, 100
Tetracycline	10, 30, 50, 100
Vancomycin	2.5

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KNOWN-SOURCE LIBRARY

Construction and Use. Fecal samples (scat) from known sources in the watershed were collected during the study period by EA Engineering personnel and shipped to the BST Laboratory at SU. *Enterococcus* isolates were obtained from known sources (e.g., human, dog, horse, deer, fox, goose, rabbit, raccoon). For Winters Run Watershed, a Library of patterns of *Enterococcus* isolate responses to the panel of antibiotics was analyzed using the statistical software CART® (Salford Systems, San Diego, CA). *Enterococcus* isolate response patterns were also obtained from bacteria in water samples collected at the monitoring stations in the basin. Using statistical techniques, these patterns were then compared to those in the Library to identify the probable source of each water isolate.

STATISTICAL ANALYSIS

We applied a tree classification method, [1]CART®, to build a model that classifies isolates into source categories based on ARA data. CART® builds a classification tree by recursively splitting the Library of isolates into two nodes. Each split is determined by the antibiotic

[1] The Elements of Statistical Learning: Data Mining, Inference, and Prediction. Hastie T, Tibshirani R, and Friedman J. Springer 2001.

variables (antibiotic resistance measured for a collection of antibiotics at varying concentrations). The first step in the tree-building process splits the Library into two nodes by considering every binary split associated with every variable. The split is chosen that maximizes a specified index of homogeneity for isolate sources within each of the nodes. In subsequent steps, the same process is applied to each resulting node until a *stopping* criterion is satisfied. Nodes where an additional split would lead to only an insignificant increase in the *homogeneity index* relative to the *stopping* criterion are referred to as *terminal* nodes.[2] The collection of *terminal* nodes defines the classification model. Each *terminal* node is associated with one source, the source isolate that is most populous among the Library isolates in the node. Each water sample isolate is identified with one specific *terminal* node and is assigned the source identity associated with that *terminal* node.[3]

Statistical analysis of bacterial concentrations was performed using Minitab Statistical Software v. 15 (Minitab, Inc., State College, PA).

Winters Run Watershed ARA Results

Known-Source Library. A 637 known-source isolate library was constructed from sources in the Winters Run Watershed. The number of unique antibiotic resistance patterns was calculated, and the known sources in the combined library were grouped into four categories: human, livestock (horse), pet (dog), and wildlife (deer, fox, goose, rabbit, raccoon) (Table 2-WIN). The library was analyzed for its ability to take a subset of the library isolates and correctly predict the identity of their host sources when they were treated as unknowns. Average rates of correct classification (ARCC) for the library were found by repeating this analysis using several probability cutoff points. The number-not-classified for each probability was determined. From these results, the percent unknown and percent correct classification (RCCs) was calculated (Table 3-WIN).

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[2] An ideal split, i.e., a split that achieves the theoretical maximum for homogeneity, would produce two nodes each containing Library isolates from only one source.

[3] The CART® tree-classification method we employed includes various features to ensure the development of an optimal classification model. For brevity in exposition, we have chosen not to present details of those features, but suggest the following sources: Breiman L, et al. *Classification and Regression Trees.* Pacific Grove: Wadsworth, 1984; and Steinberg D and Colla P. *CART—Classification and Regression Trees.* San Diego, CA: Salford Systems, 1997.

Table 2-WIN: Winters Run. Category, total number, and number of unique patterns in the known-source library.

Category	Potential Sources	Total Isolates	Unique ARA Patterns
Human	human	136	85
Livestock	horse	56	30
Pet	dog	80	56
Wildlife	deer, fox, goose, rabbit, raccoon	365	104
Total		637	275

For Winters Run Watershed, a cutoff probability of 0.70 (70%) was shown to yield an overall rate of correct classification of 87.5 % (Figure 1-WIN; Table 3-WIN). The resulting rates of correction classification (RCCs) for the four categories of sources in the Winters Run Library are shown in Table 4-WIN.

Table 3-WIN: Winters Run. Number of isolates not classified, percent unknown, and percent correct for seven (7) cutoff probabilities for Winters Run known-source isolates using the Winters Run known-source library.

Threshold	0	0.375	0.5	0.6	**0.7**	0.8	0.9
% correct	77.1%	77.1%	78.5%	78.5%	**87.5%**	90.4%	97.2%
% unknown	0.0%	0.0%	7.1%	7.1%	**23.4%**	29.5%	48.7%
# not classified	0	0	45	45	**149**	188	310

Figure 1-WIN. Winters Run Classification Model: Percent Correct versus Percent Unknown using the Winters Run library.
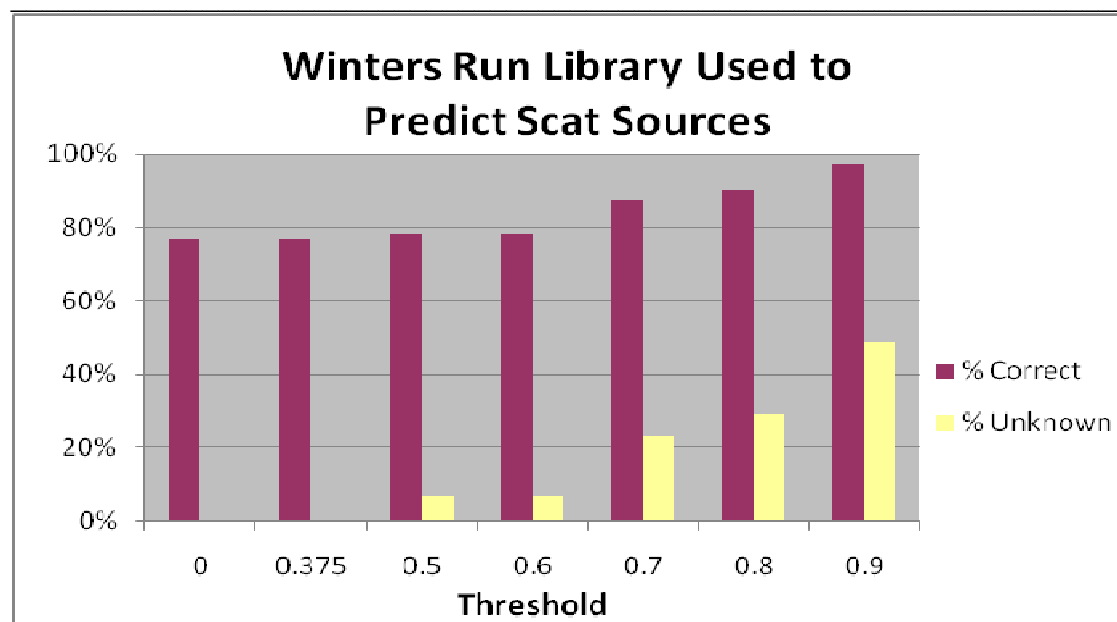

Table 4-WIN: Winters Run. Actual species categories versus predicted categories, at 70% probability cutoff, with rates of correct classification (RCC) for each category.

				Predicted			
Actual	Human	Livestock	Pet	Wildlife	Unknown	Total	RCC*
Human	117	1	1	14	3	136	88.0%
Livestock	1	28	2	25	0	56	50.0%
Pet	3	1	64	9	3	80	83.1%
Wildlife	19	17	10	101	218	365	68.7%
Total	140	47	77	149	224	637	75.1%

*RCC = Actual number of predicted species category / Total number predicted.
Example: 163 pet correctly predicted / 175 total number predicted for pet = 163/175 = 95%.

Winters Run Water Samples. Monthly monitoring from two (2) monitoring stations on Winters Run was the source of water samples. Six replicate samples were also obtained during the year. The maximum number of *Enterococcus* isolates obtained per water sample was 24, although the number of isolates that actually grew was sometimes less than 24. A total of 931 *Enterococcus* isolates were analyzed by statistical analysis. The BST results by species category, shown in Table 5-WIN, indicate that 74% of the water isolates were able to be classified to a probable host source when using a 0.70 (70%) probability threshold. The statistical analysis can also be conducted without allowing for unknowns (forcing all water isolates into a specific category). These results are also shown in Table 5-WIN.

Table 5-WIN: Winters Run. Probable host sources of water isolates by species category, number of isolates, and percent isolates classified at a cutoff probability of 70%.

Source	Count	Percent	Percent Without Unknowns
Human	193	20.7%	29.4%
Livestock	60	6.4%	9.1%
Pet	119	12.8%	18.1%
Wildlife	284	30.5%	43.3%
Unknown	275	29.5%	
Total	931	100.0%	100.0%
% classified	**70.5%**		

*Percentages may not add up to 100% due to rounding.

The seasonal distribution of water isolates from samples collected at each sampling station is shown below in Table 6-WIN.

Table 6-WIN: Winters Run. *Enterococcus* isolates obtained from water collected during the spring, summer, fall, and winter seasons for Winters Run's two (2) monitoring stations, numbers 1 and 2.

	Season				
Station	Spring	Summer	Fall	Winter	Total
1	95	72	168	66	401
2	83	72	166	67	388
11*	24	24	0	24	72
22*	0	24	46	0	70
Total	202	192	380	157	931

*Station numbers 11 and 22 represent replicate sampling for station numbers 1 and 2, respectively.

Tables 7-WIN and 8-WIN on the following pages show the number and percent of the probable sources for each monitoring station by date. Note that in Table 7-WIN below, station numbers 11* and 22* represent replicate sampling for station numbers 1 and 2, respectively.

**Table 7-WIN. Predicted Source Distribution Count by Station and Date, 70 %
Threshold Probability.**

		Predicted					
Station	*Date*	Human	Livestock	Pet	Wildlife	Unknown	Total
1	11/15/07	9	1	4	9	1	24
1	11/29/07	5	1	5	7	6	24
1	12/19/07	4	4	2	5	9	24
1	01/31/08	4	1	4	9	6	24
1	02/28/08	6	1	0	5	6	18
1	03/05/08	7	2	3	10	2	24
11*	03/05/08	7	0	4	6	7	24
1	04/29/08	3	0	3	7	11	24
1	05/13/08	2	4	4	6	8	24
11*	05/13/08	2	7	3	6	6	24
1	06/26/08	5	0	1	9	9	24
11*	06/26/08	0	2	0	15	7	24
1	08/28/08	9	0	1	8	6	24
1	09/23/08	7	1	4	9	3	24
1	11/20/08	0	0	1	4	19	24
1	12/10/08	2	2	0	5	15	24
1	12/18/08	16	1	2	4	1	24
2	11/15/07	7	2	4	10	1	24
22*	11/15/07	5	0	8	10	1	24
2	11/29/07	2	2	13	4	3	24
22*	11/29/07	5	0	9	2	6	22
2	01/31/08	4	0	0	12	8	24
2	02/28/08	3	1	0	11	4	19
2	03/05/08	2	1	2	8	11	24
2	03/27/08	1	1	1	4	4	11
2	04/29/08	2	1	7	8	6	24
2	05/13/08	3	11	2	4	4	24
2	05/22/08	3	1	2	4	14	24
2	06/26/08	4	3	1	6	10	24
22*	06/26/08	5	1	2	9	7	24
2	08/28/08	0	3	1	5	15	24
2	09/23/08	3	0	0	8	13	24
2	11/20/08	1	1	4	7	10	23
2	12/10/08	3	1	0	9	10	23
2	12/18/08	16	2	2	3	1	24
	Total	193	60	119	284	275	931

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Table 8-WIN. Predicted Source Distribution Percent by Station and Date, 70% Threshold Probability.

Station	Date	Predicted Human	Livestock	Pet	Wildlife	Unknown	Total
1	11/15/07	38%	4%	17%	38%	4%	100%
1	11/29/07	21%	4%	21%	29%	25%	100%
1	12/19/07	17%	17%	8%	21%	38%	100%
1	01/31/08	17%	4%	17%	38%	25%	100%
1	02/28/08	33%	6%	0%	28%	33%	100%
1	03/05/08	29%	8%	13%	42%	8%	100%
11*	03/05/08	29%	0%	17%	25%	29%	100%
1	03/27/08	75%	8%	0%	8%	8%	100%
1	04/29/08	13%	0%	13%	29%	46%	100%
1	05/13/08	8%	17%	17%	25%	33%	100%
11*	05/13/08	8%	29%	13%	25%	25%	100%
1	05/22/08	17%	0%	43%	30%	9%	100%
1	06/26/08	21%	0%	4%	38%	38%	100%
11*	06/26/08	0%	8%	0%	63%	29%	100%
1	07/23/08	4%	0%	0%	54%	42%	100%
1	08/28/08	38%	0%	4%	33%	25%	100%
1	09/23/08	29%	4%	17%	38%	13%	100%
1	11/20/08	0%	0%	4%	17%	79%	100%
1	12/10/08	8%	8%	0%	21%	63%	100%
1	12/18/08	67%	4%	8%	17%	4%	100%
2	11/15/07	29%	8%	17%	42%	4%	100%
22*	11/15/07	21%	0%	33%	42%	4%	100%
2	11/29/07	8%	8%	54%	17%	13%	100%
22*	11/29/07	23%	0%	41%	9%	27%	100%
2	12/19/07	29%	0%	17%	38%	17%	100%
2	01/31/08	17%	0%	0%	50%	33%	100%
2	02/28/08	16%	5%	0%	58%	21%	100%
2	03/05/08	8%	4%	8%	33%	46%	100%
2	03/27/08	9%	9%	9%	36%	36%	100%
2	04/29/08	8%	4%	29%	33%	25%	100%
2	05/13/08	13%	46%	8%	17%	17%	100%
2	05/22/08	13%	4%	8%	17%	58%	100%

NOTE: In Tables 8-WIN above and below, station numbers 11 and 22* represent replicate sampling for station numbers 1 and 2, respectively.

Table 8-WIN. Predicted Source Distribution Percent by Station and Date, 70% Threshold Probability (Continued).

		Predicted					
Station	Date	Human	Livestock	Pet	Wildlife	Unknown	Total
2	06/26/08	17%	13%	4%	25%	42%	100%
22*	06/26/08	21%	4%	8%	38%	29%	100%
2	07/23/08	25%	0%	25%	21%	29%	100%
2	08/28/08	0%	13%	4%	21%	63%	100%
2	09/23/08	13%	0%	0%	33%	54%	100%
2	11/20/08	4%	4%	17%	30%	43%	100%
2	12/10/08	13%	4%	0%	39%	43%	100%
2	12/18/08	67%	8%	8%	13%	4%	100%
	Total	21%	6%	13%	31%	30%	100%

Figure 2-WIN: Winters Run Watershed relative contributions by probable sources of *Enterococcus* contamination.
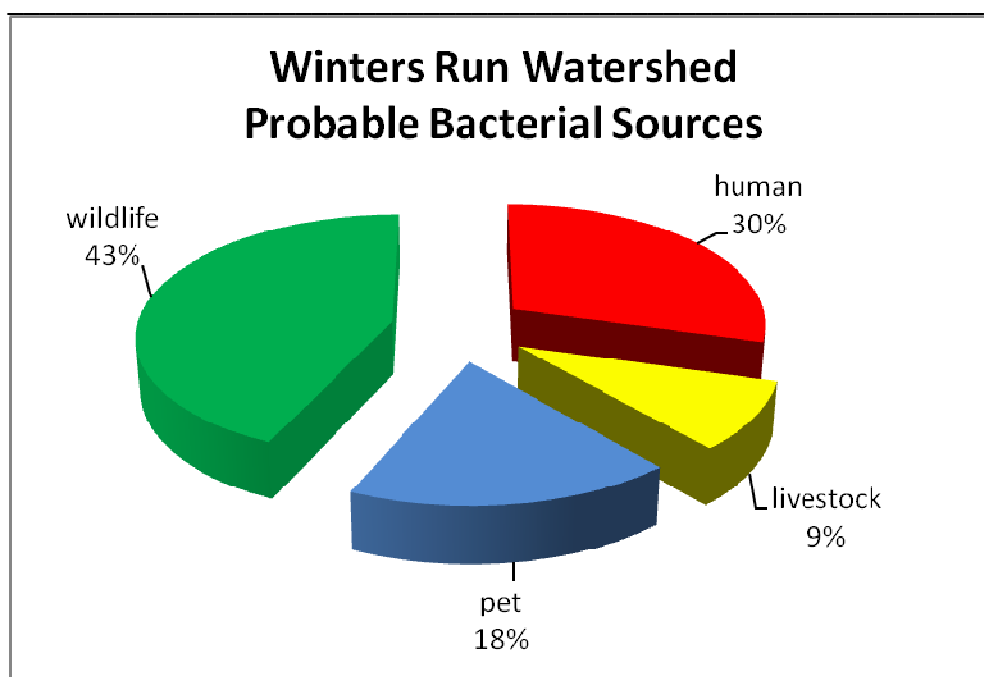
Table 9-WIN. Predicted Source Distribution Percent by Station and Date, Comparison of Replicate Samples.

Station	Date	Predicted Human	Livestock	Pet	Wildlife	Unknown	Total
1	03/05/08	29%	8%	13%	42%	8%	100%
11*	03/05/08	29%	0%	17%	25%	29%	100%
1	05/13/08	8%	17%	17%	25%	33%	100%
11*	05/13/08	8%	29%	13%	25%	25%	100%
1	06/26/08	21%	0%	4%	38%	38%	100%
11*	06/26/08	0%	8%	0%	63%	29%	100%
2	11/15/07	29%	8%	17%	42%	4%	100%
22*	11/15/07	21%	0%	33%	42%	4%	100%
2	11/29/07	8%	8%	54%	17%	13%	100%
22*	11/29/07	23%	0%	41%	9%	27%	100%
2	06/26/08	17%	13%	4%	25%	42%	100%
22*	06/26/08	21%	4%	8%	38%	29%	100%

Winters Run ARA Summary

The use of ARA was successful for identification of probable bacterial sources in the Winters Run Watershed. When water isolates were compared to the library and potential sources predicted, 71% of the isolates were classified as to category by statistical analysis. The highest RCC for the Library was 88% for human, followed by 83% for pet. Wildlife had an RCC of 69%, while livestock had an RCC of 50%.

The largest category of potential sources in the watershed as a whole was wildlife (43% of classified water isolates), followed by human and pet (30% and 18%, respectively). The remaining potential source contribution was for livestock (9%) (Fig. 2-WIN).

The predicted sources from replicate water samples, as expressed in percentage values, are somewhat variable (Table 9-WIN). A comparison of replicate samples for major vs. minor source contributors shows much greater consistency. Variability in predicted sources as expressed in percentages is not surprising, as each set of predictions is derived from 24 randomly selected isolates. This illustrates that ideally, conclusions concerning probable sources of contamination at specific water sampling sites should be based upon multiple samples over time as opposed to individual water samples collected on a given date.

Winters Run Watershed Bacterial Density Results

Descriptive Statistics and Distribution. Together, the 34 water samples collected from stations 1 and 2 on the unnamed tributary of Winters Run had a mean density of *Enterococcus* bacteria of 582 colony forming units per 100 ml of samples (CFU/100 ml) and a standard deviation of 1,587 CFU/100 ml (Table 10-Win). Individually, station 1 had a mean and standard deviation of 451 CFU/100 ml and 288 CFU/100 ml, respectively (Table 10-WIN). In contrast, station 2 had a higher mean and standard deviation of 713 CFU/100 ml and 470 CFU/100 ml, respectively (Table 10-Win). Monthly bacterial density can be seen in Table 10.

Table 10-WIN. Winters Run. Descriptive statistics for bacterial densities (CFU/100 ml) of water samples collected from both stations 1 and 2.

Stations	N	Mean	Std Dev	Min	Max
1 and 2	34	582	1,587	5	8,000

Table 10-WIN. Winters Run. Stations 1 and 2 descriptive statistics for bacterial density (CFU/100 ml).

Station	N	Mean	Std Dev	Minimum
1	17	451	288	5
2	17	713	470	5

The bacterial density data followed a lognormal distribution (Figures 3-WIN and 4-WIN). The geometric mean of the data was 92 CFU/100 ml.
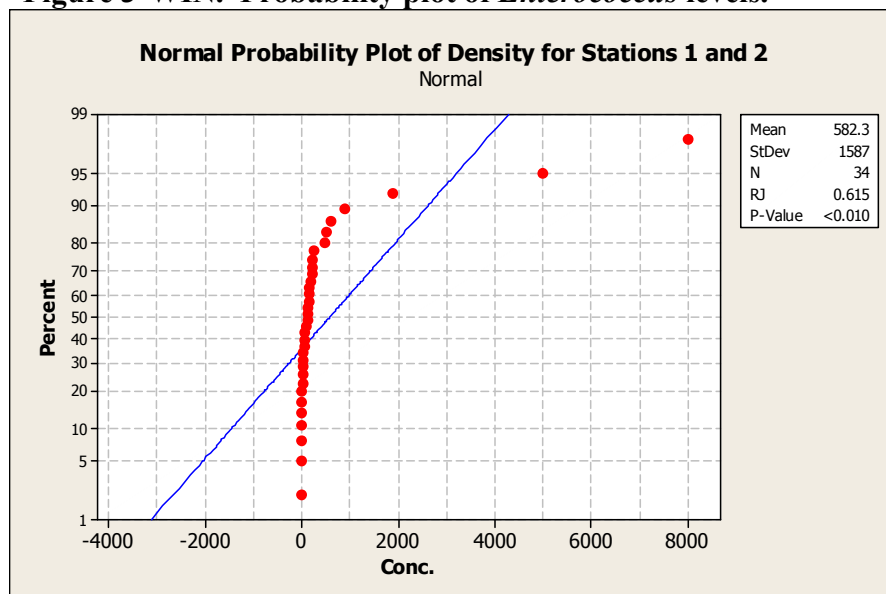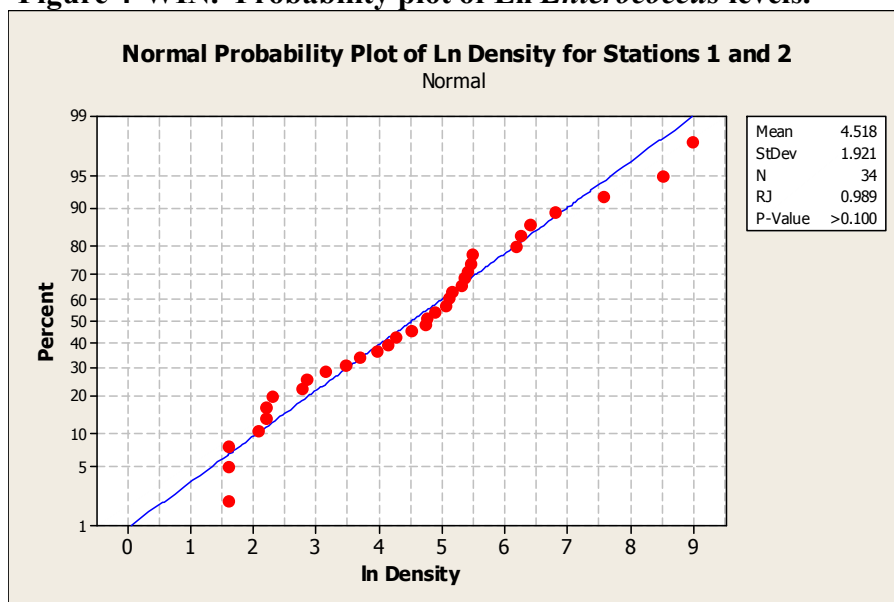
Figure 3-WIN. Probability plot of *Enterococcus* levels.

Figure 4-WIN. Probability plot of Ln *Enterococcus* levels.

Replicates Sampling and Rain Events. Six replicate samples were collected during the project period, three (3) from each of stations 1 and 2. A one-way ANOVA analysis performed to test whether the mean bacterial densities of replicate samples differed from that of the original samples indicated that there was not a significant difference between the means (p = 0.602; f = 0.66) (Data not shown but available upon request).

Samples were collected on six days following a rain event, while 29 samples were collected following a dry period. A one-way ANOVA analysis performed to test whether the mean bacterial densities of rain event samples differed from that of dry-period samples found that rain event samples were significantly higher (Data not shown but available upon request).

Enterococcus **Bacterial Density Summary**

Bacterial indicator organisms to determine water quality for freshwater recreation in Maryland can be either *Enterococcus* or *E. coli*. *Enterococcus* is the organism used for recreational marine waters.

Maryland water quality standards are established in the Code of Maryland Regulations and depend on type of water use and frequency of use and (shellfish harvesting, bathing beach, or water contact recreation). Therefore, whether a single sample collected during this project exceeded any water quality standard would depend on how often the water was used and what type of water contact occurred.

Statistical analysis of water samples collected from both stations on the unnamed tributary of Winter Run analysis found that water samples collected from both stations had a mean of 582 CFU of *Enterococcus* bacteria per 100 ml, with densities ranging from 5 CFU/100 ml to the high of 8,000 CFU/100 ml. The highest *Enterococcus* levels were found after rain events, although high levels (> 510 CFU/100 ml) were found following dry periods as well. In general, higher bacterial levels were seen during the warmer spring and summer months.

Table 11-WIN, on the following page, shows the *Enterococcus* density of each sample and replicate, by date and monitoring station, and indicates whether sampling was performed following a rain event.

Note for Table 11-WIN (below), Station numbers 11* and 22* are replicate samples for station numbers 1 and 2, respectively. Est = Estimated count, not based on ideal number of colony-forming units.

Table 11-WIN. Winters Run. Bacterial density (CFU/100 ml) from membrane filtration of water samples.

Sample Date	Rain Event	Station	CFU/100 ml
11.15.07	Rain	1	>5,000 Est
		2	>8,000 Est
		22*	>8,000 Est
11.29.07		1	9 Est
		2	10
		22*	14
12.19.07	Rain	1	70
		2	62
01.31.08		1	9 Est
		2	16
02.28.08		1	5 Est
		2	9 Est
03.05.08		1	130
		11*	160
		2	157
03.27.08	Rain	1	9 Est
		2	5 Est
04.29.08	Rain	1	600
		2	1,900
05.13.08	Rain	1	163
		11*	180
		2	240
05.22.08	Rain	1	32
		2	220
06.26.08		1	173
		11*	195
		2	210
		22*	350
07.23.08		1	480
		2	230
08.28.08		1	200
		2	40
09.23.08		1	113
		2	17
11.20.08		1	117
		2	23
12.10.08		1	510
		2	900
12.18.08		1	53
		2	90

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